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PRESERVATION OF MAMMALIAN TISSUES AND ORGANS BY FREEZING.(U)
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Contract No. ¹⁵ NO 0014-73-C-0156 *new*

⁹ FINAL REPORT.

⁶ Preservation of Mammalian Tissues and Organs by Freezing,
by

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¹¹ 1 Sep 77

¹² 10 P.

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September 1, 1977

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During the past five years, this research contract has been maintained at no cost to the government for the purpose of obtaining continued use of two pieces of major equipment purchased under a prior ONR contract and considered essential to the continuation of the study. Rather than to recite in detail the large number of specific projects carried out during this interval of time, the following paragraphs summarize the conclusions drawn. Concurrent with the end of this contract, we feel that we have arrived at last at a clear understanding of the nature of freezing injury and the mechanism of action of cryoprotective agents.

Prior to 1950, most of the emphasis in cryobiology was on the crystallization of ice in tissues, based on the assumption that freezing injury to living cells was largely the result of mechanical damage from ice (Luyet and Gehenio, 1940). Attempts to achieve artificial cryoprotection were based either on modification of ice growth through additives or more commonly, by attempting to minimize ice crystallization through ultra-rapid freezing and thawing. The latter approach has been uniformly without success. Very rapid freezing rates produce intracellular ice which is apparently uniformly lethal (Meryman, 1957). Achieving freezing and thawing rates sufficiently rapid to prevent ice crystallization all together is beyond the range of current technology.

In 1949 Smith, Parkes, and Polge reported the ability of glycerol to protect bull sperm and human red cells (Smith 1950) from freezing injury. The subsequent classic papers of Lovelock (1953a, 1953b) demonstrated that freezing injury was not a direct consequence of ice formation but resulted from the concentration of solutes with the freezing out of extracellular water. Lovelock showed that in red cells injury resulted when the extracellular salt concentration exceeded 0.8 molar regardless of the presence or absence of glycerol or the freezing temperature necessary to produce that salt concentration. Lovelock interpreted this as evidence that membrane components were being denatured by the elevated salt concentration. He presumed that the protective qualities of glycerol were the result of a simple anti-freeze effect. Adding large quantities of glycerol reduced the amount of ice formed

at any temperature and thereby reduced the concentration of salt to a tolerable level. This so-called colligative theory of cryoprotection has been generally accepted since that time although more recently some aspects of the theory have been questioned (Mazur, Leibo and Miller, 1971).

Lovelock's supposition that cells were damaged by elevated salt concentration has not been supported by subsequent evidence. For example, we have shown that cells are injured at roughly the same freezing temperature even though they may be suspended in non-electrolytes instead of salt (Meryman, 1967). We have also shown that cell injury does not appear to result directly from the concentration of solutes but from osmotic dehydration of the cell (Meryman, 1968). Our evidence suggest that injury from osmotic dehydration is related not to dehydration per se but to volume reduction of the cell beyond a minimum critical volume (Meryman, 1970). Our studies of plant ecells (Williams and Meryman, 1970), red cells (Meryman and Douglas, 1972), platelets (Kahn and Meryman, 1973) and frog muscle (Meryman and Bakry, 1973) suggest that there is a resistance to cell shrinkage that results in an osmotic pressure gradient across the membrane. With increasing osmotic dehydration this pressure gradient increases until there is an alteration in membrane permeability and an influx of extracellular solution. Associated with this influx there may be changes in the plasma membrane which initially appear to be reversible but which subsequently progress to irreversibility and cell lysis (Meryman and Hornblower, 1972).

The membrane changes resulting from hypertonic stress can be postponed to higher osmolalities or prevented entirely by an event which enables the cell to shrink less than would be predicted. Four means of achieving this end have been observed in naturally freeze resistant tissues (Meryman, 1974): 1) The solute content of the cell can be increased so that a higher external osmolality is necessary to achieve the same volume reduction. 2) Cell water can be rendered osmotically inactive, presumably by being structured at interfaces, so that a larger proportion of free water must be lost by the cell before it reaches critical minimum volume, 3) Plasma membrane can become permeable to external solutes permitting an influx of external solution to forestall volume reduction. 4) The cell can acquire an unusual resistance to shrinkage and maintain a volume greater than the minimum critical despite an increase in external osmolality. We have also observed cells in nature which, when they become freeze resistant, are able to tolerate a smaller critical volume, apparently by losing membrane lipid to acquire

a lesser membrane area (Meryman et al., 1977). The conclusion drawn from these many studies is that freezing injury from extracellular ice is the result of osmotic dehydration of the cell leading to disruption of the plasma membrane on a mechanical basis.

Many investigators have found that increasing the rate of cooling can increase the recovery of cells after freezing and thawing. This implies that there is some destructive process which requires time and that achieving a low, stabilizing temperature more rapidly may not afford sufficient time for this process to go to completion. (This would appear to contradict our mechanical stress hypothesis of freezing injury, a contradiction that more recent studies appear to have resolved.) As the cooling rate is further increased, recovery again decreases. This has been attributed to the development of intracellular ice (Leibo and Mazur, 1971). These authors also showed that the cooling rates at which recovery increased and then decreased again varied from one species of cell to another.

Cryoprotectants shift these curves to lower cooling rates (Rapatz and Luyet, 1965), suggesting that the agents are in some way causing the deleterious effects of freezing to occur at a slower rate or somehow protecting the cells against the stresses of freezing and dehydration. We (Meryman, 1974) showed that almost without exception, such agents in aqueous solution strengthen hydrophobic forces and act as stabilizers. However, a series of experiments designed to measure the extent to which cryoprotectants stabilized cells against dehydration injury failed to demonstrate any stabilizing effect. Red cells, very slowly frozen in the presence or absence of a variety of agents (ethanol, methanol, glycerol, dimethyl sulfoxide (DMSO), sucrose and polyvinyl pyrrolidone (PVP)) hemolyzed in proportion to the osmolality of extracellular, non-penetrating solute, regardless of the presence or absence of a cryoprotectant, its concentration or the temperature of freezing. This demonstrated unequivocally that, for red cells at least, cryoprotectants did not alter the relationship between cell dehydration (volume) and injury (Meryman et al., 1977).

How, then, can we explain the fact that increasing the rate of cooling increases cell recovery and that cryoprotectants shift this effect to slower cooling rates? If the agent does not affect the point of injury, but only the rate at which it is achieved, then the agent must be modifying whatever the rate process is during rapid cooling.

Mazur (1963) has examined the kinetics of water loss from cells during rapid freezing, explaining the development of intracellular ice on the basis of a lag in water loss from the cell leading to excessive supercooling and the

nucleation of intracellular ice. This hypothesis, which Mazur has developed extensively, also provides an explanation for the increased recovery at higher cooling velocities. As the cooling rate increases, less time is available for water loss from the cell so that cell dehydration and volume reduction lag behind what they would be at equilibrium. If the cooling rate is fast enough, the cell may not be sufficiently dehydrated to suffer injury. At still faster cooling rates with even less cell dehydration, the cell may freeze internally and recovery again fails.

Since cryoprotective agents such as sucrose and PVP are nominally restricted to the extracellular space, any effect on cell recovery must then be exerted extracellularly. Both Mazur and we (Mazur, 1977; Meryman *et al.*, 1977) have speculated that these extracellular cryoprotectants may be increasing the viscosity of the extracellular unfrozen solution, further impeding the movement of water from the cell to the ice crystal.

The following model for freezing injury and cryoprotection can therefore be proposed. At slow rates of cooling, a few ice crystals form and grow throughout the cell suspension, concentrating extracellular solutes and causing osmotic dehydration of the cells. The resulting reduction in cell volume leads to stresses in the cell plasma membrane which ultimately result in collapse of the membrane. As the rate of cooling is increased there is insufficient time for water to move from the cell interior to the ice, so that the dehydration of the cell is less at any given temperature than it would be if the cell interior had reached vapor pressure equilibrium with the ice. If the lag is sufficient, the cell may not have been dehydrated to a damaging extent before the temperature has fallen sufficiently to prevent further water diffusion altogether, and cell recovery becomes possible. If the cooling is even more rapid, the cell may suffer very little dehydration and its contents will be so dilute that intracellular ice will form, destroying intracellular structures.

Cryoprotectants influence recovery in two ways, both physical chemical in nature. First, on a colligative basis they can lower the freezing point and reduce the amount of ice formed, and therefore the extent of cell dehydration. Second, they can decrease the rate of water diffusion from cell interior to the ice, thus increasing the lag in cell water loss at higher cooling velocities.

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